Effect of creatine monohydrate supplementation on relative serum level of IL-6 and IL-18 following neonatal hypoxia ischemia in male albino mouse

Shahid Iqbal[1], Muhammad Ali[2] and Furhan Iqbal[1]*

[1]Institute of Pure and Applied Biology, Zoology Division, Bahauddin Zakariya University, Multan, Pakistan
[2]Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan

Abstract: IL-6 has been reported to have neuroprotective effects against cerebral ischemia while IL-8 is a pro inflammatory cytokine structurally related to interleukin-1 family. In the present study, we tried to determine whether 2% Creatine monohydrate supplementation for variable duration influence the IL-6 and 18 concentrations in the serum of male albino mouse following right common carotid artery ligation and hypoxia (8% oxygen) for 25 minutes. Our result revealed that serum concentration of IL6 (P=0.0001) as well as IL-18 (P=0.003) were significantly higher in mice supplemented with creatine monohydrate for 15 weeks than in male albino mice on normal rodent diet following hypoxic ischemic insult indicating that long term creatine monohydrate supplementation up regulates the IL-6 and IL-18 concentrations triggering the neuroinflammatory and neuroprotective responses.

Keywords: Creatine, Hypoxia ischemia, interleukin 6, interleukin 18.

INTRODUCTION

During delivery or gestation the accidental asphyxia of infants or perinatal hypoxia ischemia (HI) is a common cause of neurological deficit and delayed behavioral and cognitive deficits (Kaltschmidt *et al.,* 1994). Cerebral hypoxia means reduction of O_2 content in blood and ischemia characterized any insult that leads to reduction in cerebral blood flow partially or completely (Budd, 1998). Neonatal HI is a major cause of morbidity and mortality in infants and is occurs around 2 to 4/1000 full term births. Between 20 to 50% of asphyxiated newborns die within the neonatal period and up to 25% of the survivors exhibits morbidity, such as cerebral palsy, mental retardation and epilepsy (Paula *et al*., 2009).

Creatine and phosphocreatine play a vital role in the transmission and storage of phosphate bound energy (Bianchi *et al*., 2000). Liver, pancreas and kidney synthesize approximately 2% of the total body creatine while 98% of creatine is taken with food. Creatine is naturally present in meat and to some extent in green leaved vegetables. 60% of creatine found in the body is in the form of creatine phosphate (Iqbal *et al*., 2013; Iqbal *et al*., 2014; Iqbal *et al*., 2015). Creatine shows protective effects in acute neuronal damage models, such as traumatic brain injury (Sullivan *et al*., 2000) and stroke (Zhu *et al*., 2004). In neurodegenerative animal models, creatine supplementation has been revealed to reduce oxidative stress, improve brain functioning and attenuate neuronal disintegration (Andreassen *et al*., 2001; Dedeoglu *et al*., 2003; Hersch *et al*., 2006). Despite of extensive studies on creatine and its neuroprotective effects, little information is available regarding the effect of creatine monohydrate supplementation on blood chemistry following hypoxic ischemic encephalopathy.

Interleukin 6 (IL-6) is a cytokine originally recognized as a T-cell derived factor regulating B-cell differentiation and growth (Hirano *et al*., 1986) exerting numerous biological effects *in vivo* and its down regulation underlies several disease processes (Xu *et al*., 1997). IL-6 is mainly involved in the inflammatory reactions, which contribute to both injury and repair process after cerebral ischemia (Suzuki *et al*., 2009). The pro inflammatory cytokine IL-18 is a member of interleukin-1 family (Okamura *et al*., 1995; Bazan *et at*., 1996). It is produced by immune as well as by non immune cells such as macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells and synovial fibroblasts (Stoll *et al*., 1997, 1998; Udagawa *et al*., 1997; Conti *et al.,* 1997., Pizarro *et al*., 1999; Prinz *et al*., 1999; Gracie *et al*., 1999; Matsui *et al*., 1997). IL-18 effects are complex and pleiotropic including T cells activation and natural killer cells in autoimmune disorders (Reddy, 2004). It has been reported that IL-18 is involve in delayed neuroinflammatory events after HI brain injury (Wheeler *et al*., 2003; Jander *et al*., 2002; Allan and Rothwell, 2001). Aim of this study was to demonstrate whether 2% Creatine monohydrate (Cr) supplementation influence neuroprotective role of IL-6 and 18 in male albino mouse following right common carotid artery ligation and hypoxia (8% oxygen) for 25 minutes.

**Corresponding author:* e-mail: furhan.iqbal@bzu.edu.pk

MATERIALS AND METHODS

Male albino mice (N=30) were used during the present study. Breeding pairs of mice were purchased from Veterinary Research Institute, Ghazi road, Lahore and maintained in cages provided with wood chips and cotton piece at the core animal facility at the Bio park of Bahauddin Zakariya University, Multan (Pakistan). Albino mice were kept in separate cages with free access to water and standard rodent diet throughout the experimental duration. Room temperature was maintained at 22±1°C and relative humidity was 50±10%. The light/dark rhythm was 14:10. All the experimental procedures and mouse handling protocols were approved by the ethical committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan.

Murine model of hypoxia-ischemia encephalopathy

On postnatal day 10 of pups, corresponding to the brain development of 40 weeks gestational age in human fetus (Romijn *et al*., 1991; Allahyar *et al*., 2015), 3% isoflurane inhalation was used for anesthetization of pups. A right lateral neck incision was made and polypropylenedalcon USP 6 suture was used for the ligation of right common carotid artery. During surgery pups were placed on a hot plate, maintaining 36°C temperature. The whole surgical process was accomplished within 10 minutes after that pups were placed for 30min with in their dams for nursing and recovery. Then mice were kept for 25min in a hypoxic chamber maintaining with steady flow of 8% oxygen balanced with Nitrogen. The hypoxic chamber was placed on hot plate for maintain the 36°C ambient temperature inside the chamber. Pups were taken back to their mothers after hypoxic insult, for recovery. Following weaning on postnatal day 20, mice were separated from their parents and housed in individual cages. Control groups were fed with standard rodent diet while experimented treatments received 2% creatine monohydrate supplemented diet (Ssniff, Germany) for 8, 12 and 15 weeks

IL-6 and IL-18 demonstrations

At the end of each diet supplementation experiment, blood was sampled from retro-orbital sinus under Isoflurane inhalation. Blood was centrifuged for ten minutes at 13000 rpm and plasma was separated for biochemical analysis of interleukin. IL-18 was determined in serum by using enzyme-linked immuno absorbent assay (ELISA) and IL-8 diagnostic Kit (MBL, Japan). While IL- 6 was measured by enzyme-linked immunoabsorbent assay, using Mouse 1L-6 antibody pair (Invitrogen, California. Catalog No. CMC0063) following the instruction of manufactures.

STATISTICAL ANALYSIS

All the data is expressed as Mean ± Standard deviation. Statistical package Minitab (version 13, Pennsylvania) was used for the analysis of results. 2 sample t-test was applied to compare ILs concentrations between creatine monohydrate treated and untreated groups following hypoxia ischemia encephalopathy.

RESULTS

Our results indicated that supplementation of 2% creatine monohydrate following HI did not affected IL-6 concentrations after 8 (P=0.096) and 12 (P=0.778) weeks but IL-6 concentration was significantly higher (P=0.0001) in 15 weeks creatine monohydrate treated male albino mice as compared to their creatine monohydrate untreated control group indicating a time dependent change in IL-6 concentrations following hypoxic ischemic insult (fig. 1). An exactly similar trend was observed for IL-18 concentrations having no significant effect of creatine monohydrate supplementation after 8 (P=0.953) and 12 (P=0.895) weeks but significant increase in IL-18 concentration (P=0.003) was observed in creatine monohydrate treated group as compared to male albino mouse on normal rodent diet after brain damage (fig. 2).

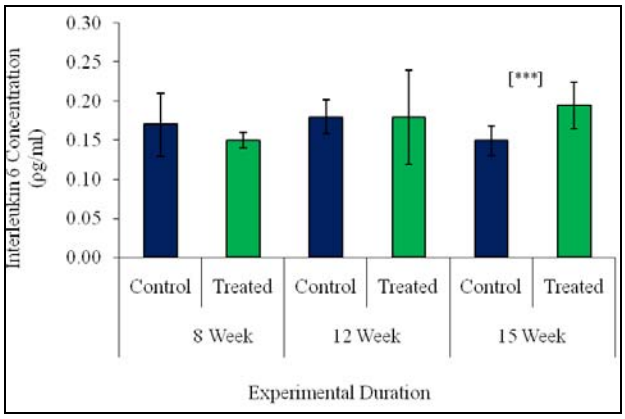

Fig. **1**: Comparison of IL-6 concentrations between creatine monohydrate treated (for 8, 12 and 15 weeks) and untreated male albino mice.
P<0.001 = highly significantly different (***)

DISCUSSION

Neuroinflammation has been extensively investigated following hypoxia-ischemia in both humans and animal models and all these studies have reported a robust elevation of cytokines in the CNS (Morganti-Kossmann *et al*., 2007; Ziebell and Morganti-Kossmann 2010; Frugier *et al*., 2010; Xia *et al*., 2010; Macrez *et al*., 2011). In order to find out the potential effect of creatine monohydrate supplementation on interleukin concentrations in serum, we supplemented male albino mouse with 2% creatine monohydrate in diet for variable time duration (8, 12 and 15 weeks) following neonatal asphyxia on postnatal day 10.

IL-6 might directly involved in infarct pathogenesis, or is simply a marker of central nervous system's inflammatory injury and it has been reported previously that permanent focal cerebral ischemia results in a dramatic increase in brain IL-6 activity in rats (Wang *et al*., 1995), and transient global ischemia in gerbils (Saito *et al*., 1996). Infants' identified with hypoxia ischemia encephalopathy has also represented elevated concentrations of IL-6, IL-1b and tumor necrosis factor- α in the cerebrospinal fluid when compared to control infants (Aly *et al*., 2006). We have also observed that there is a gradual increase in serum IL-6 levels from 8 to 15 weeks in hypoxia ischemia insulted male albino mice. IL-6 concentrations were higher in Cr treated males with the highest concentrations observed in 15 week treatment group (fig. 1) indicating that IL-6 concentration increases with the duration of creatine monohydrate supplementation. IL-6 is known to have both neurotrophic and neuroprotective affects and its increased production is might be involved in the repair process and this cytokine is playing an important role as endogenous inhibitor of neuronal death after ischemic brain damage (Chiesa *et al*., 2003; Loddick *et al*., 1998).

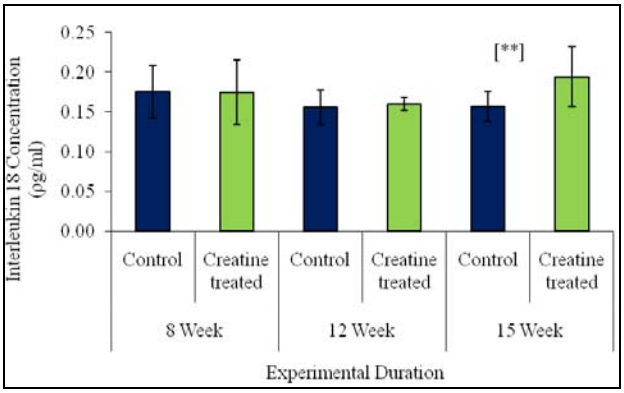

Fig. **2**: Comparison of IL 18 concentrations between creatine monohydrate treated (for 8, 12 and 15 weeks) and untreated male albino mice.
P<0.001 = significantly different (**)

IL-18 is a pro-inflammatory cytokine of the IL-1 family and its receptors are expressed in the CNS where they participate in neuro-inflammatory/neurodegenerative processes but also influence homeostasis and behavior suggested (Alboni *et al*., 2010). We have also observed that there is a gradual increase in serum IL-18 levels from 8 to 15 weeks in hypoxia ischemia insulted male albino mice. IL-18 concentrations were higher in creatine monohydrate treated males with the highest concentrations observed in 15 week treatment group (fig. 2) indicating that IL-18 concentration increases with the duration of creatine monohydrate supplementation. It has been reported that after cerebral ischemia IL-18 level is increased but the functional role of IL-18 in cerebral ischemia is unidentified (Allan and Rothwell, 2001). Probably IL-18 is contributing to delay neuronal injury and/or repair (Liu *et al*., 1993; Zhang *et al*., 1998; Touzani *et al*., 1999). Hedtjärn *et al*., 2002, had suggested that IL-18 is participated in immature brain's white matter pathologies as in IL-18-deficient mice, white matter injury was significantly reduced when compared with wild mice after HI). Further evidences were documented by an activation of IL-18 in the brain of mice that underwent thromboembolic stroke (Abulafia *et al*., 2009), or enhancement of IL-18 levels in the juvenile hippocampus of mice after hypoxia-ischemia (Qiu *et al*., 2007). IL-18 appears to be a negative factor in brain recovery from asphyxia as it is known to delay the inflammatory response indicating that Il6 and 18 cytokines are playing variety of roles in neuronal mortality as well as in rescuing them. Recently we have conducted a similar study in female albino mice to determine the effect of variable concentration of creatine monohydrate on IL-6 and IL-18 concentrations following HI insult (Allahyar *et al*., 2014). Results indicated a high concentration of IL-6 and lower concentration of IL-18 in 3% Cr supplemented mice indicating that creatine is regulating the levels of both cytokines to protect the brain from HI damage.

CONCLUSION

We concluded that IL-6 and 18 are actively involved during the process of hypoxia ischemia mediated brain damage and in the recovery process. Concentration of both interleukins increased in a time dependent manner with maximum levels observed after 15 weeks of creatine monohydrate supplementation indicating that creatine monohydrate is up regulating the IL6 and 18 depended cascades in order to neutralize the brain damage on post natal day 10 in male albino mice as both interleukins are known to be associated with neuroprotection.

REFERENCES

Abulafia DP, de Rivero Vaccari JP, Lozano JD, Lotocki G, Keane RW and Dietrich WD (2009). Inhibition of the inflammasome complex reduces the inflammatory response after thromboembolic stroke in mice. *J. Cereb. Blood Flow Metab.*, **29**: 534-544.

Alboni S, Cervia D, Sugama S and Conti B (2010). Interleukin 18 in the CNS. *J. Neuroinflamm.*, **7**: 7-9.

Allahyar R and Iqbal F (2014). Creatine monohydrate supplemented diets affects serum inflammatory cytokine levels (IL-6 and IL-18) in female albino mice following neonatal hypoxic ischemic encephalopathy. *Pak. J. Zool.*, **46**(5): 1311-1315.

AllahYar R, Akbar A and Iqbal F (2015). Creatine monohydrate supplementation for 10 weeks mediates neuroprotection and improves learning/memory following neonatal hypoxia ischemia encephalopathy in female albino mice. *Brain Res*., **1595**: 92-100.

Allan SM and Rothwell NJ (2001). Cytokines and acute neurodegeneration. *Nat. Rev. Neuros*., **2**: 734-744.

Aly H, Khashaba MT, El-Ayouty M, El-Sayed O and Hasanein BM (2006). IL-1beta, IL-6 and TNF-alpha and outcomes of neonatal hypoxic ischemic encephalopathy. *Brain Dev*., **28**: 178-182.

Andreassen OA, Dedeoglu A, Ferrante RJ, Jenkins BG, Ferrante KL and Thomas M (2001). Creatine increase survival and delays motor symptoms in a transgenic animal model of Huntington's disease. *Neurobiol. Dis.*, **8**: 479-491.

Bazan JF, Timans JC and Kastelein RA (1996). A newly defined interleukin-1? *Nature*., **379**: 591-591.

Bianchi MC, Tosetti M, Fornai F, Alessandri MG, Cipriani P, Vito GD and Canapicchi R (2000). Reversible brain creatine deficiency in two sisters with normal blood creatine level. *Ann. Neurol.*, **47**: 511-513.

Budd SL (1998). Mechanisms of Neuronal Damage in Brain Hypoxia/Ischemia: Focus on the Role of Mitochondrial Calcium Accumulation. *Pharmacol. Ther*., **80**(2): 203-229.

Chiesa C, Pellegrini G, Panero A, De Luca T, Assumma M, Signore F and Pacifico L (2003). Umbilical cord interleukin-6 levels are elevated in term neonates with perinatal asphyxia. *Eur. J. Clin. Invest*, **33**: 352-358.

Conti B, Jahng JW, Tinti C, Son JH and Joh TH (1997). Induction of interferon-gamma inducing factor in the adrenal cortex. *J. Biol. Chem*., **272**: 2035-2037.

Dedeoglu A, Kubilus JK, Yang L, Ferrante KL, Hersch SM and Beal MF (2003). Creatine therapy provides neuroprotection after onset of clinical symptoms in Huntington's disease transgenic mice. *J. Neurochem.*, **85**: 1359-1367.

Frugier T, Morganti-Kossmann MC, O'Reilly D and McLean CA (2010). In situ detection of inflammatory mediators in post mortem human brain tissue after traumatic injury. *J. Neurotrauma*., **27**: 497-507.

Gracie JA, Forsey RJ, Chan WL, Gilmour A, Leung BP, Greer MR, Kennedy K, Carter R, Wei XQ, Xu D, Field M, Foulis A, Liew FY and McInnes IB (1999). A proinflammatory role for IL-18 in rheumatoid arthritis. *J. Clin. Invest,* **104**: 1393-1401.

Hedtjärn M, Mallard C, Arvidsson P and Hagberg H (2005). White matter injury in the immature brain: Role of interlukin-18. *Neur. Scie. Lett.*, **373**: 16-20.

Hersch SM, Gevorkian S, Marder K, Moskowitz C, Feigin A and Cox M (2006). Creatine in huntington disease is safe, tolerable, bioavailable in brain and reduces serum 8OH2'dG. *Neurol*., **66**: 250-252.

Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashiwamura S, Nakajima K, Koyama K, Iwamatsu A, Tsunasawa S, Sakiyama F, Matsui H, Takahara Y, Taniguchi T and Kishimoto T (1986). Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature*, **324**: 73-76.

Iqbal S, Nazir N, Gillani QUA, Akbar A and Iqbal F (2013). Effect of creatine monohydrate supplementation on various hematological and serum biochemical parameters of male albino mice following neonatal hypoxia-ischemia encephalopathy. *Scient. World J.*, http://dx.doi.org/10.1155/ 2013/286075.

Iqbal S, Ali M, Akbar A and Iqbal F (2014). Effects of dietary Creatine supplementation for 8 weeks on neuromuscular coordination and learning in male albino mouse following neonatal hypoxic ischemic insult. *Neurolog. Sci.*, doi: 10.1007/s10072-014-2041-9

Iqbal S, Ali M and Iqbal F (2015). Long term creatine monohydrate supplementation, following neonatal hypoxic ischemic insult, improves neuromuscular coordination and spatial learning in male albino mouse. *Brain Res.*, **1603**: 76-83.

Jander S, Schroeter M and Stoll G (2002). Interleukin-18 expression after focal ischemia of the rat brain: association with the late-stage inflammatory response. *J. Cereb. Blood Flow Metab.,* **22**: 62-70.

Kaltschmidt C, Kaltschmidt B, Neuman H, Wekerle H and Baeuerle PA (1994). Constitutive NF-kappa B activity in neurons. *Mol. Cell. Biol*., **14**: 3981-3992.

Liu T, McDonnell PC, Young PR, White RF, Siren AL, Hallenbeck JM, Barone FC and Feuerstein GZ (1993). Interleukin-1_ mRNA expression in ischemic rat cortex. *Stroke,* **24**: 1746-1750.

Loddick SA, Turnbull AV and Rothwell NJ (1998). Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. *J. Cereb. Blood Flow Metab.*, **18**(2): 176-179.

Macrez R, Ali C, Toutirais O, Le Mauff B, Defer G, Dirnagl U and Vivien D (2011). Stroke and the immune system: from patho physiology to new therapeutic strategies. *Lancet Neurol*., **10**: 471-480.

Matsui K, Yoshimoto T, Tsutsui H, Hyodo Y, Hayashi N, Hiroishi K, Kawada N, Okamura H, Nakanishi K and Higashino K (1997). Propionibacterium acnes treatment diminishes CD4+ NK1.1+ T cells but induces type I T cells in the liver by induction of IL-12 and IL-18 production from Kupffer cells. *J. Immunol.,* **159**: 97-106.

Morganti-Kossmann MC, Satgunaseelan L, Bye N and Kossmann T (2007). Modulation of immuneresponse to head injury. *Injury*, **38**: 1392.

Okamura H, Tsustsui H, Komatsu T, Yatsudo M, Hakura A, Tanimoto T, Torogoe K, Okura T, Nukada Y, Hattori K, Akita K, Namba M, Tanebe F, Konoshi K, Fukuda S and Kurimoto M (1995). Cloning of a new cytokine that induces IFN-g production by T cells. *Nature*, **378**: 88-91.

Paula S, Vitola AS, Greggio S, Paula D, Pamela Billig Mello PB, Lubianca JM, Xavier LL, Humberto Holmer Fiori HH and Dacosta JC (2009). Hemispheric brain injury and behavioral deficits induced by severe Neonatal hypoxia-ischemia in rats are not attenuated by

intravenous administration of human umbilical cord blood cells. **65**(6): 631-635.

Pizarro TT, Michie MH, Bentz M, Woraratanadharm J, Smith MF, Jr Foley E, Moskaluk CA, Bickston SJ and Cominelli F (1999). IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: Expression and localization in intestinal mucosal cells. *J. Immunol.,* **162**: 6829-6835.

Prinz M and Hanisch UK (1999). Murine microglial cells produce and respond to interleukin-18. *J. Neurochem.,* **72**: 2215-2218.

Qiu L, Zhu C, Wang X, Xu F, Eriksson PS, Nilsson M, Cooper-Kuhn CM, Kuhn HG and Blomgren K (2007). Less neurogenesis and inflammation in the immature than in the juvenile brain after cerebral hypoxia-ischemia. *J. Cereb. Blood Flow Metabol*., **27**: 785-794.

Reddy P (2004). Interleukin-18: Recent advances. *Curr. Opin. Hematol.,* **11**: 405-410.

Romijn HJ, Hofman MA and Gramsbergen A (1991). At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? *Early Hum. Dev.,* **26**(1): 61-67.

Saito K, Suyama K, Nishida K, Sei Y and Basile AS (1996). Early increases in TNF-oc, IL-6, IL-I i3 levels following transient cerebral ischemia in gerbil brain. *Neurosci. Lett.,* **206**: 149-152.

Stoll S, Jonuleit H, Schmitt E, Muller G, Yamauchi H, Kurimoto M, Knop J and Enk AH (1998). Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC derived IL-18 enhances IL-12-dependent Th1 development. *Eur. J. Immunol.,* **28**: 3231-3239.

Stoll S. Muller G, Kurimoto M, Saloga J, Tanimoto T, Yamauchi H, Okamura H, Knop J and Enk AH (1997). Production of 11-1 8 (IFN-y-Inducing Factor) Messenger RNA and Functional Protein by Murine Keratinocytes. *J. Immunol.,* **159**: 298-302.

Sullivan PG, Geiger JD, Mattson MP and Scheff SW (2000). Dietary supplement creatine protects against traumatic brain injury. *Ann. Neurol.*, **48**: 723-729.

Suzuki S, Tanaka K and Suzuki N (2009). Ambivalent aspects of interleukin-6 in cerebral ischemia: Inflammatory versus neurotrophic aspects. *J. Cereb. Blood Flow Metabol.*, **29**: 464-479.

Touzani O, Boutin H, Chuquet J and Rothwell N (1999). Potential mechanisms of interleukin-1 involvement in cerebral ischemia. *J. Neuroimmunol*., **100**: 203-215.

Udagawa N, Horwood NJ, Elliott J, Mackay A, Owens J, Okamura H, Kurimoto M, Chambers TJ, Martin TJ and Gillespie MT (1997). Interleukin-18 (interferon-gamma-inducing factor) is produced by osteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-gamma to inhibit osteoclast formation. *J. Exp. Med.,* **185**: 1005-1012.

Wang X, Yue TL, Young PR, Barone FC and Feuerstein GZ (1995). Expression of interleukin-6, c-fos and zif268 mRNAs in rat ischemic cortex. *J. Cereb. Blood Flow Metabol.,* **15**: 166-171.

Wheeler DR, Boutin H, Touzani O, Luheshi NG, Takeda K and Rothwell JN (2003). No role for interleukin-18 in acute murine stroke-induced brain injury. *J. Cereb. Blood Flow Metabol.,* **23**: 531-535.

Xia W, Han J, Huang G and Ying W (2010). Inflammation in ischaemic brain injury: Current advances and future perspectives. *Clin. Exp. Pharmacol. Physiol*., **37**: 253-258.

Xu GYi, Yu H, Hong J, Stahl M, McDonagh T, Kay LE and Cumming DA (1997). Solution structure of recombinant human interleukin-6. *J. Mol. Biol*., **268**: 468-481.

Zhang Z, Chopp M, Goussev A and Powers C (1998). Cerebral vessels express interleukin 1beta after focal cerebral ischemia. *Brain Res*., **784**: 210-217.

Zhu S, Li M, Figueroa BE, Liu A, Stavrovskaya IG, Pasinelli P, Beal MF, Brown RHJR, Kristal BS, Ferrante RJ and Friedlander RM (2004). Prophylactic creatine administration mediates neuroprotection in cerebral ischemia in mice. *J. Neurosci.*, **24**: 5909-5912.

Ziebell JM and Morganti-Kossmann MC (2010). Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. *Neurotherapeut*., **7**: 22-30.